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Role of Incretins and DPP-4 Inhibitors in the Regulation of Adipokine Secretion

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Abstract

Background: The hallmarks of type 2 diabetes are both insulin resistance and insulin insufficiency. Obesity is a high risk factor of type 2 diabetes. As an endocrine organ, adipose tissue can secrete a large number of adipokines that regulate metabolism and inflammation. It was shown that pro-inflammatory adipokines are elevated in obesity associated with insulin resistance. Obesity has been shown to induce ER stress in liver causing insulin resistance. Our previous work shows that ER stress deteriorates adipokines secretion from adipocytes. Incretin analogues and DPP-4 inhibitors are now used in the clinic to treat patients suffering from type 2 diabetes. Incretins can regulate lipid synthesis and glucose uptake in adipocytes. However, no data are available regarding whether incretins affect the production and secretion of adipokines from adipose tissue. The aim of this study was therefore to test if incretins and DPP-4 inhibitors regulate the secretory function of adipocytes.

Material and Methods: After differentiation, 3T3-L1 adipocytes were incubated in the presence of GLP-1(7-36), GLP-1(9-36), GLP-1 receptor agonist (Exendin-4) and DPP-4 inhibitor, Sitagliptin (DPP-4i) individually or in combinations under ER stress or homeostasis. The secretion of adipokines into the supernatant was measured by multiplexing. The induction of ER stress was confirmed by Western blotting.

Results: ER stress elevated the accumulation of the proinflammatory cytokines KC (keratinocyte chemoattractant) and IL-6 (Interleukin-6) but reduced adiponectin concentrations. GLP-1(7-36), Exendin-4 and GLP-1(9-36) affected the accumulation of adipokines under homeostasis and ER stress in a similar manner. They increased adiponectin and KC under homeostasis but reduced KC under ER stress. DPP-4i increased pro-inflammatory cytokines KC and IL-6 under homeostasis.

Discussion and conclusion: In the present study, we show that incretins and the inhibitor Stagliptin regulate the secretory function of adipocytes. Importantly, Stagliptin increases pro-inflammatory cytokines without changing secretion of adiponectin or leptin. This could explain why patients treated with Stagliptin have a higher risk for infection. Therefore, a possible immune-modulatory role of DPP-4i should be taken into account for clinical use of these compounds.

1 Introduction

1.1 Diabetes

1.1.1 The history of diabetes

Since a long time diabetes is recognized as a systemic complex disease. As summarized in the education web site of the *Canadian Diabetes Association* [2], as early as 1552 BC, Egyptian physician Hesy-Ra mentioned a disease that is associated with "passing of too much urine". In 120 AD the word "diabetes" was first used by Aretaeus, a Greek, to describe the symptoms of patients having a large amount of urine flowing. In the 17th century, Professor Thomas Willis of Oxford University used the smell and taste of urine samples from patients to determine whether they were diabetic. If the urine had a sweet taste, he would diagnose the patient with diabetes mellitus. In 1901, American pathologist Eugene Opie established a connection between the failure of the islets of Langerhans in the pancreas and the occurrence of diabetes. In 1921, Dr. Frederick Banting led the discovery of insulin that was extracted from pancreas and he was awarded the Nobel Prize in Physiology or Medicine in 1923. Since the discovery of insulin, diabetes has become a treatable disease. In 1936, Sir Harold Himsworth of the University College Hospital in London found that diabetes falls into two types based on "insulin insensitivity". This discovery later lead to the diabetes classifications of type 1 and type 2 in 1959. Diabetes mellitus now is defined as a syndrome of impaired carbohydrate, fat and protein metabolism characterized by high blood glucose and dyslipidemia. It is caused by either absolute lack of insulin or by decreased sensitivity of insulin target tissues in combination with relative hypoinsulinaemia [3].

1.1.2 Classification of diabetes

According to the American Diabetes Association there are four types of diabetes mellitus by etiological classification: type 1 diabetes, type 2 diabetes, other specific types of diabetes (genetic defects in insulin action, drug or chemical-induced diabetes, infection-induced diabetes and so on) and gestational diabetes [4] (see Table. 1).

1. Type 1 diabetes 1.1. Immune-mediated 1.2. Idiopathic
2.Type 2 diabetes
3. Other specific types 3.1. Genetic defects of β-cell function 3.2. Genetic defects in insulin action 3.3. Diseases of the exocrine pancreas 3.4. Endocrinopathies 3.5. Drug- or chemical-induced 3.6. Infections 3.7. Uncommon forms of immune-mediated diabetes 3.8. Other genetic syndromes sometimes associated with diabetes
4. Gestational diabetes mellitus (GDM)

Table 1: Etiological classification of diabetes mellitus. Modified from [5].

1.1.3 Type 2 diabetes

90% of all diabetes cases are type 2 diabetes [6]. Proper regulation of normal blood glucose levels depends on the balance of sufficient insulin production/secretion relative to insulin sensitivity in liver, muscle and adipose tissue. The hallmark of type 2 diabetes is the combined occurrence of both insulin resistance and insulin insufficiency. Diabetes eventually

develops when under prolonged persisting insulin resistance the pancreatic β-cells fail to adapt, not producing sufficient insulin for normal regulation [7].

The major risk factor for type 2 diabetes is obesity [8]. However, a conclusive mechanistic link between obesity, insulin resistance and the development of the metabolic syndrome is still missing. There are genetic and environmental components in the events leading to β-cell failure. Chronic exposure of pancreatic islets to elevated nutrients induces β-cell dysfunction and death. It was concluded that β-cell dysfunction is a consequence of 'glucolipotoxicity' rather then exposure to a single nutrient alone [9].

1.1.4 Insulin and insulin resistance

1.1.4.1 Insulin and insulin signal transduction

The production of insulin exclusively occurs in pancreatic β-cells [10]. Mature insulin accumulates within vesicles in the cytoplasm of pancreatic β-cells and is secreted upon glucose stimulation. The main function of insulin is to lower circulating blood glucose levels, which is mainly mediated by insulin-stimulated glucose uptake into skeletal muscle (and to a minor part adipose tissue and even kidney) and by insulin-induced suppression of glucose release (gluconeogenesis and glycogenolysis) from the liver. Inhibition of free fatty acid (FFA) release from adipose tissue and stimulation of protein synthesis in skeletal muscle are other important metabolic functions of insulin.

When bound to insulin, insulin receptors autophosphorylate and transfer the signal down stream by phosphorylating and attracting insulin receptor substrates (IRS). Tyrosine-phosphorylated IRS contain many docking sites for downstream signalling components such as phospatidylinositol 3-kinase (PI3-K). Protein kinase B (PKB) and protein kinase C (PKC) isoforms λ and ζ, which are downstream factors of PI3-K, are then activated and contribute to regulating glucose homeostasis [11] (Figure 1).

1.1.4.2 Insulin resistance

Insulin resistance was discovered 60 years ago in 1939 by Sir Himsworth. He set forth the hypothesis that insulin deficiency should not be the only reason for the development of diabetes. He concluded that insensitivity of the tissues to insulin may also contribute to the process [12]. 10 years later it was shown that normal or elevated insulin levels can be present in diabetic patients [13]. Today, insulin resistance is defined as insufficient action of insulin in target tissues and it is one of the major factors leading to the development of type 2 diabetes [14].

At the molecular level insulin resistance is due to blocked insulin signal transmission [15, 16]. In particular, phosphorylation of IRS on specific serine residues inhibits tyrosine phosphorylation of IRS by decreasing affinity for the insulin receptor kinase, resulting in reduced transmission of the insulin signal. Many factors contribute to this blocking process such as cytokines like tumour necrosis factor-α (TNF-α) [17], FFAs [18], c-Jun N-terminal kinase (JNK) [19] and nuclear factor-kappa B (NF-κB) [20].

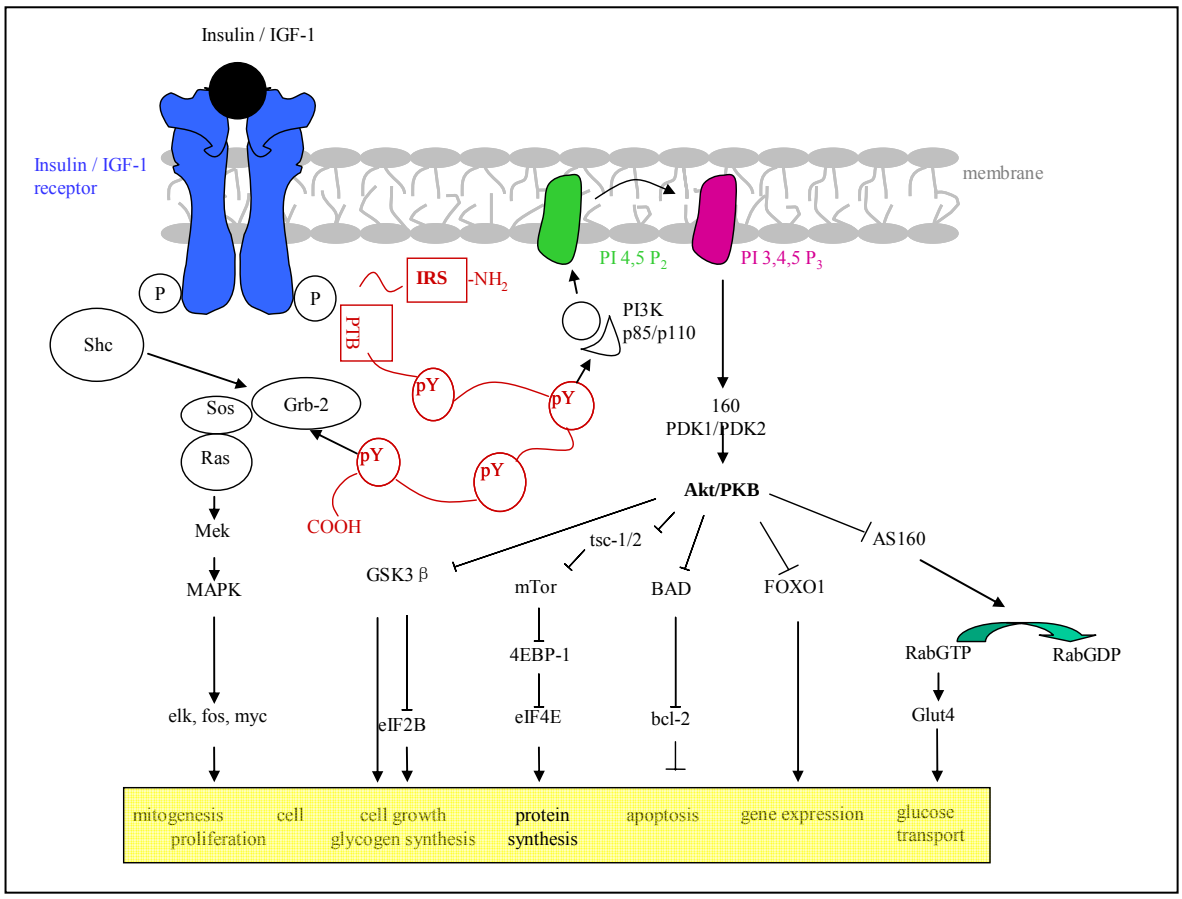

Figure 1: The insulin signalling network. **Kindly provided by Markus Niessen and Stephan Wüest.**

1.2 Adipose tissue

Adipose tissue (AT) is a loose connective tissue containing lipid-laden adipocytes, macrophages, fibroblasts, blood cells, endothelial cells and adipose precursor cells [21]. Adipocytes comprise around 35% to 70% of adipose tissue's mass in adults. The lipid droplets of adipocytes contain a mixture of neutral fats, triglycerides, fatty acids, phospholipids and cholesterol.

1.2.1 Classification and function of adipose tissue

According to the biochemical and functional characteristics, AT is classified into two types: brown adipose tissue (BAT) and white adipose tissue (WAT) [22].

BAT is rich in mitochondria and lysosomes and responsible for thermogenesis by transferring energy from food into heat [23]. In the neck and interscapular region of newborn, BAT is well developed, but the distribution in childhood is limited and only a small amount is present in adults. This may be because the need for thermogenesis is reduced due to clothing and indoor life [24].

For a long time, it was believed that the function of WAT was limited to storing excess energy as triacylglycerol (TG) in large unilocular droplets, and its release in form of free fatty acids for oxidation by skeletal muscle and other tissue [24]. The discovery of leptin and of its secretion from adipose tissue in 1994 extended the understanding of adipose tissue function [25]. In addition to energy storage, the endocrine function of WAT is now well recognized and known to play a central role in whole body homeostasis [26]. At the same time, the finding that adipoytes themselves express receptors for most adipokines reveals that there are both local and systemic controls employed for the regulation of metabolic balance [27].

1.2.2 Adipokines

As already indicated above, adipose tissue functions as an endocrine organ to regulate the metabolic balance of the body by secreting a great number of bioactive mediators, which are

called adipokines [16, 28]. Table 2 summarizes the main adipokines and their known functions. Among these, we pick some factors for more in-depth description, because they have close relationship to the development of diabetes as described below.

1.2.2.1 Leptin

Leptin was discovered in 1994 [25]. It is produced by differentiated adipocytes [29]. The plasma concentration of leptin is directly related to the severity of obesity [30]: an increased leptin level is found when fat mass is increased.

Leptin regulates food intake (appetite) and energy homeostasis [31, 32]. It improves insulin sensitivity of liver and skeletal muscle and it was shown that mice lacking white adipose tissue suffer from severe hepatic and muscle insulin resistance with increased TG storage in these tissues [33]. Furthermore, leptin also plays an important role in pancreatic β-cells. It was shown that leptin inhibits insulin secretion in lean animals. However, when body fat increases, leptin protects β-cells and improves β-cell function [34]. The concept of "adipo-insular axis" was introduced based on the finding that insulin stimulates leptin biosynthesis and secretion from adipose tissue and it is a classical endocrine feedback loop [35]. However, in obesity leptin fails to reduce body weight, although both leptin and leptin receptor levels are elevated, suggesting "leptin resistance".

1.2.2.2 Adiponectin

In 1995, an adipose-secreted protein was first identified and named adiponectin, Acrp 30 or adipoQ [36]. Its concentration in circulation is reduced under obesity [37], insulin resistance [38] and type 2 diabetes [39].

There are three forms of adiponectin in circulation: trimers, hexamers and high-molecular weight (HMW) 12-18 mers [40]. The HMW are the biologically active forms of the hormone [41] and they are related to insulin sensitivity and development of the metabolic syndrome [42]. Two similar case studies have suggested that people with high adiponectin concentration have lower risk to develop type 2 diabetes compared to those with low adiponectin concentration [43], [44]. In addition to improving insulin sensitivity, adiponectin can reduce

the inflammatory response induced by tumor necrosis factor-α (TNF-α) [45]. On the other hand, TNF-α and interleukin-6 (IL-6) repress adiponectin expression in human adipocytes[46]

1.2.2.3 IL-6

IL-6 is a cytokine produced by several cell types such as monocytes, fibroblasts, endothelial cells [47], adipocytes [48] and skeletal muscle cells [49]. Under normal condition, adipose tissue contributes to 15-30% of circulating IL-6 [50]. However, since adipose tissue contains a number of cell types other than adipocytes, the IL-6 secreted from adipose tissue may be derived from monocytes/macrophages, or endothelial cells [51].

IL-6 participates in many physiological events. It acts as a differentiation factor for B cells promoting their differentiation into antibody producing cells [52]. It has been reported that IL-6 induces proliferation of hybridoma/plasmacytoma/myeloma cells, IL-2 production, cytotoxic T cell differentiation and neuronal differentiation. It regulates the immune system, the hematopoietic system and controls inflammatory response [47].

IL-6 plays different roles in insulin action and insulin resistance. Studies have shown that IL-6 elevates insulin-dependent glucose uptake and glucogen synthesis in skeletal muscle. In adipocytes, effects of IL-6 are complex and context dependent. Long term studies indicated that IL-6 represses insulin signalling by reducing expression of IRS1, thereby impairing insulin-stimulated glucose transport [53]. However, infusion of IL-6 at physiological concentrations increased adipose tissue glucose uptake in humans [54]. In hepatocytes IL-6 induces insulin resistance [55]. A study by Ellingsgaard and co-workers indicated that IL-6 regulates pancreatic α-cell mass and β-cell function [56]. It also regulates expression of proglucagon mRNA and the secretion of glucagon.

1.2.2.4 TNF-α

TNF-α is a proinflammatory cytokine. It is produced by numerous cell types, but mainly by macrophages and lymphocytes. In WAT, both adipose cells and cells of the stromal vascular fraction can produce TNF-α . Studies have shown that the expression of TNF-α in adipose tissues is elevated in obesity. Upon weight loss, TNF-α is decreased and improvement of

insulin sensitivity is observed [57]. Both TNF-α and TNF-α receptor-deficient mice are protected from obesity-induced insulin resistance under high fat diet [58] suggesting this cytokine may molecularly link obesity and insulin resistance [59].

1.2.3 Obesity and insulin resistance

Although the so called body mass index (BMI) overestimates fatness in people who are muscular or athletic, it is still used to indicate if people are overweight, obese, underweight or normal. The World Health Organization (WHO) calls a person obese if he or she has a BMI > 30. Obesity results when energy intake exceeds energy expenditure. Adipose tissue mass expands by increasing size of existing fat cells (hypertrophy) and increasing the fat cell number (hyperplasia) [21]. Excess energy is initially stored through hypertrophy and cells can become up to four times larger than normal due to excess accumulation of lipids. Only when the existing fat cells reach a critical size, hyperplasic growth of adipocytes occurs [61]. Among the risk factors that are believed to contribute to the development of insulin resistance, obesity is the most important. More than 80% of subjects with type 2 diabetes are overweight [62]. However, not all obese subjects develop type 2 diabetes.

1.2.3.1 Altered production of adipokines and insulin resistance

A hallmark of obesity is chronic low-level systemic inflammation [63]. Under obesity, circulating levels of inflammatory markers and the expression of inflammation-related adipokines generally rises (adiponectin, which has anti-inflammatory action is an exception). Altered adipokine production contributes to insulin resistance. For example, increased levels of TNF-α activate IKKβ leading to phosphorylation of IRS1 on serine and blocking of insulin signal transduction [64]. Suppressors-of-cytokine-signalling (SOCS) proteins which can be elevated by cytokines under obesity were shown to inhibit insulin signalling by preventing the binding of IRS to the tyrosine-phosphorylated insulin receptor [65]. Moreover, the change of adipokine secretion under obesity not only influences insulin sensitivity in adipocytes but also affects other organs of the body. For example, elevated IL-6 and TNF-α play a role in development of non-alcoholic fatty liver disease (NAFLD) that contributes to hepatic insulin resistance [66-68].

1.2.3.2 Endoplasmic reticulum (ER) stress, the UPR and insulin resistance

The Endoplasmic reticulum (ER) is a membrane–surrounded intracellular compartment and stretches from the nuclear envelop in all eukaryotic cells [69]. It is the site of synthesis, folding and sorting of transmembrane and secretory proteins [70]. The ER is an indispensible compartment for lipid bilayer assembly and the storage of calcium ions (Ca^{2+}) [71, 72].

Under conditions that impair ER function, proteins are unable to fold properly and accumulate in the ER lumen, this causes ER stress. To deal with ER stress, cells have evolved a special coping response called unfolded protein response (UPR) (reviewed by [73]). The UPR maintains ER homeostasis by increasing the protein folding capacity through increasing the size of the ER and inducetion of ER-resident molecular chaperones as well as reducing loading of the ER through suppression of transcription and translation. The ER clears the unfolded proteins by ER-associated degradation (ERAD) [74].

There are three transmembrane proteins acting as ER sensors. They are type I transmembrane protein kinase endoribonuclease inositol-requiring protein-1 (IRE1), protein kinase-like ER kinase (PERK) and type II transmembrane protein activating transcription factor 6 (ATF6) (reviewed by [75]). Under physiological conditions, IRE1, PERK and ATF6 are inhibited when bound to the ER chaperone glucose-regulated protein 78 (Grp78, also called Bip). Under ER stress, Grp78 is released from these three sensors, resulting in oligomerization of IRE1 and PERK and translocation of ATF6 to Golgi complex and activation.

The IRE1 pathway regulates chaperone production, ERAD and hepatic lipogenesis [76]. It also recruits tumour necrosis factor receptor–associated factor 2 (TRAF2) and activates c-Jun N-terminal kinase (JNK) [77]. Activation of PERK phosphorylates eIF2α and results in downregulation of overall translation [78]. The ATF6-dependent pathway induces the expression of genes encoding molecular chaperones like *Grp78* and *Grp94* (Figure 2).

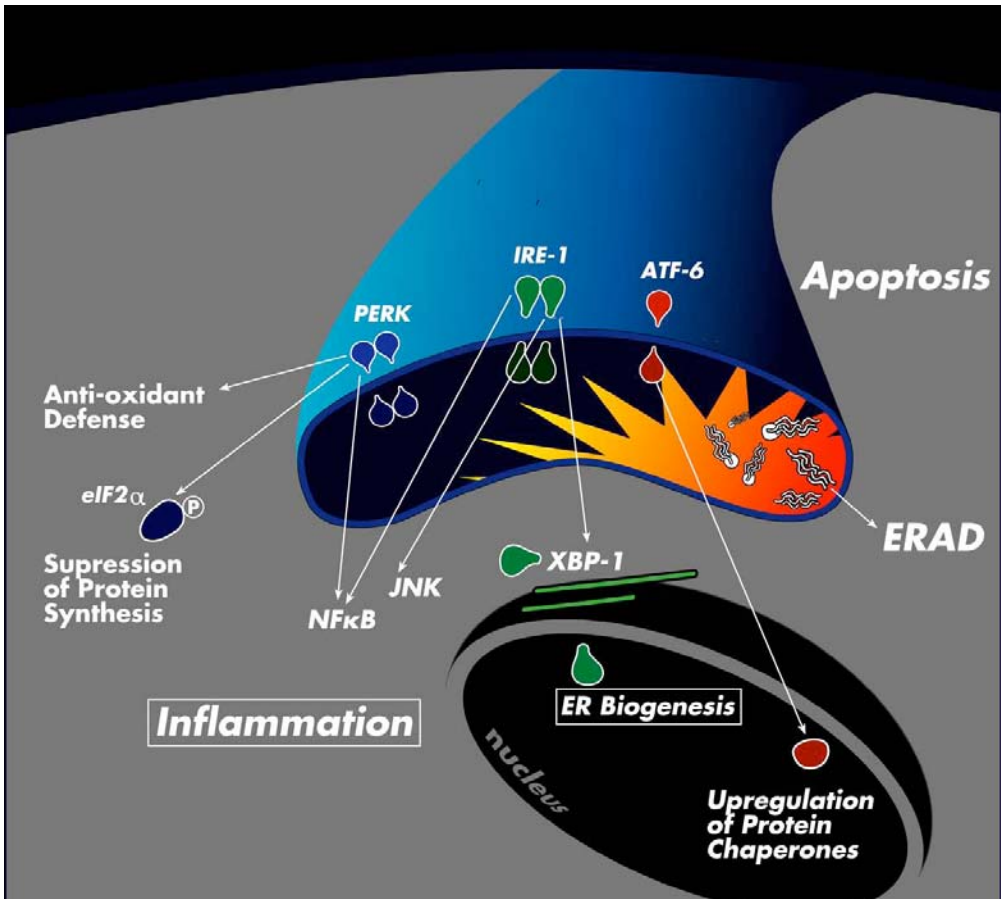

Figure 2: Schematic representation of the UPR. Adapted from [1].

In 2004, Ozcan et al. reported that ER stress links obesity and type 2 diabetes. These authors showed that ER stress occurs in liver and fat of high fat diet fed *ob/ob* mice. Activation of the UPR in response to ER stress was shown to inhibit IRS1 via JNK-dependent serine phosphorylation leading to reduced insulin sensitivity and development of insulin resistance in liver [77]. Our unpublished results show that ER stress not only impairs insulin signalling at different levels in adipocytes (insulin receptor, IRS and PKB (Figure 3)) but also alters the secretion of adipokines (elevates IL-6 and decreases leptin (Figure 4)) which are known to contribute to systemic inflammation and insulin resistance.

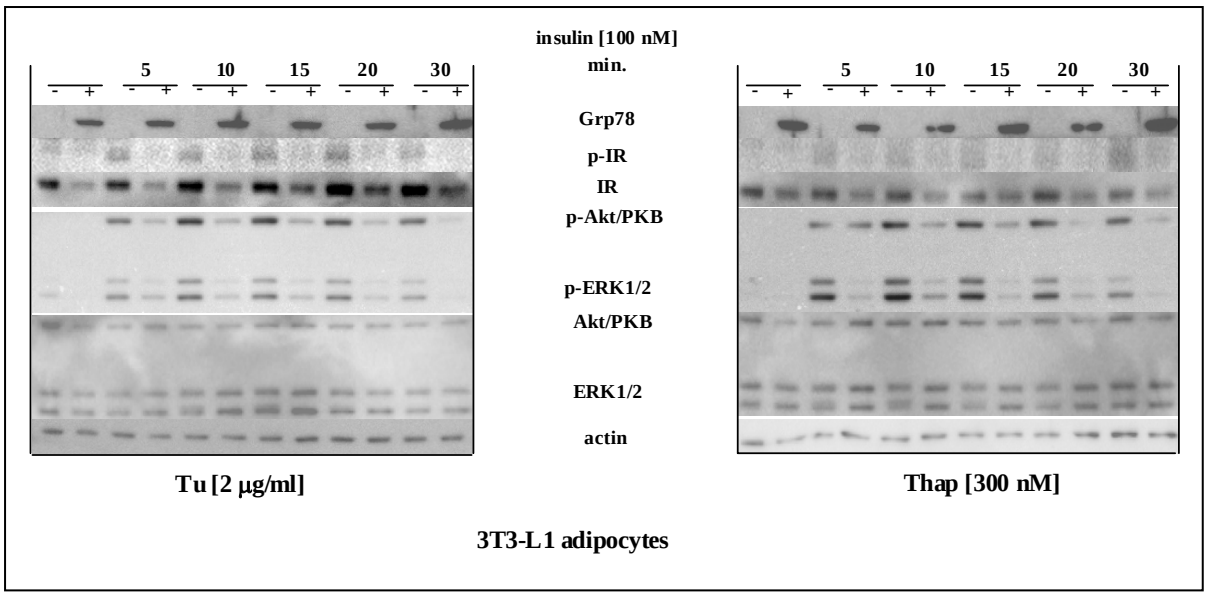

Figure 3: ER stress represses insulin signalling at different levels in 3T3-L1 adipocytes. Tunicamycin (Tu) or Thapsigargin (Thap) were included in the culture medium of 3T3-L1 adipocytes for 24 h. Before insulin (100nM) stimulation, cells were starved for 2 h. The stimulation was 5, 10, 15, 20 or 30 min. Cells were lysed afterwards. The Western blot shows expression and activity state of the IR, Akt/PKB, ERK1/2. Expression of actin was assessed as a control. The induction of ER stress was monitored using an antibody against Grp78.

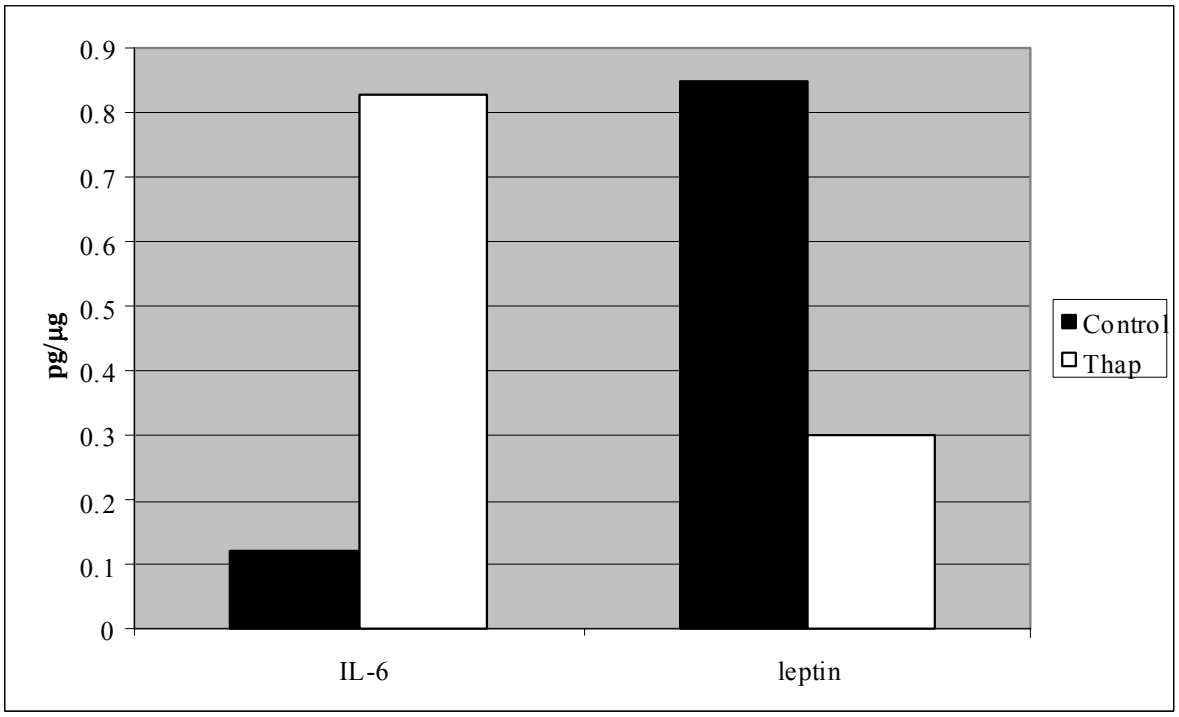

Figure 4: 3T3-L1 adipocytes secrete more IL-6 but less leptin under ER stress. 3T3-L1 adipocytes were incubated in the presence of thapsigargin (Thap, 300nM) for 16 hours. After three washes with PBS, Kreb's Ringer buffer containing 1% BSA but without substance was added to the cells for 90 min. Supernatants were collected and the accumulated IL-6 and leptin were measured by mutiplex analysis. Data from one representative experiment are shown relative to cellular protein content.

1.3 Incretins

The history of the incretin concept can be traced back to one century ago [79]. Early in 1902, Bayliss and Starling showed that acid extracts of intestinal mucosa contain a factor that stimulates exocrine secretion of pancreas and they named it secretin [80]. This report represents the birth of gastrointestinal endocrinology and challenged the traditional "nerves doctrine" that the functions of the body were controlled solely by nerves. After the report of secretin, numerous experiments have been performed to test the effect of it on fasting or elevated blood glucose levels of animal or humans. In 1932, La Barre for the first time used the term *incretin* to indicate the substance extracted from the upper gut mucasa, which produces hypoglycemia and does not stimulate pancreatic exocrine secretion [81]. In 1964, with radio-immunologic techniques the incretin effect had been proved. It became obvious that the insulin secretory responses after oral glucose ingestion exceeded those elicited by the intravenous administration of an equal amount of glucose [82], [83]. In 1970, glucose-dependent insulinotropic polypeptide (GIP) was first isolated by Brown [84] [85]. In 1985, another intestinal peptide glucagon-like peptide-1 (GLP-1) was identified during the cloning of pre-proglucagon [86].

1.3.1 GIP

GIP is a 42-aminoacid hormone and synthesised in duodenal and jejunal enteroendocrine K cells in the proximal small bowel [87]. The secretion of GIP follows in response to oral glucose or mixed meals. It has weak effects on gastric acid secretion but shows potent insulinotropic action in human beings [88]. In addition to enhancing insulin release, GIP was shown to stimulate proinsulin gene transcription and translation [89]. It also stimulates differentiation, proliferation and survival of pancreatic β-cells [90]. Moreover, it was shown that GIP can reduce hepatic glucose production [91], promote glucose uptake in isolated muscle [92] and adipocytes [93]. GIP has also been shown to reduce glucagon-stimulated lipolysis [94] and to stimulate the synthesis and secretion of lipoprotein lipase in rat adipose tissue explants [95]. The finding that GIP receptor-deficient ($GIPR^{-/-}$) mice were resistant to the development of obesity when on a high-fat diet [96] supports the view that GIP plays a crucial role in the development of obesity due to chronic over nutrition.

1.3.2 GLP-1

The biologically active forms of GLP-1 are GLP-1(7-36) [97] and GLP-1(7-37) [98]. Also GLP-1(7-37) has insulinotropic activity [98], but GLP-1(7-36) is the most active isoform [99]. Food intake in the gut causes GLP-1 production and release from small intestinal L-cells. It stimulates insulin secretion and slows gastric emptying [100]. Like GIP, GLP-1 also can promote pancreatic β-cell growth and survival [101], [102].

GLP-1 receptors can be found on many cells throughout the body, especially β-cells, liver, and neurons [103]. GLP-1 acts directly through the cAMP → protein kinase A (PKA) pathway to enhance and sensitize β-cells to glucose-stimulated insulin secretion [104]. GLP-1 (or its agonist exendin-4) also increases pancreatic β-cell survival, leading to an increase of β-cell mass. PKA-mediated activation of the transcription factor cAMP responding element binding protein (CREB) plays an important role in GLP-1 action in β-cells by increasing the expression of the glucose transporter (GLUT)-2, insulin, glucokinase, and PDX-1 [105]. CREB phosphorylation increased the induction of IRS2 in islets. Tyrosine phosphorylated IRS2 activates the phosphoinositide-3 (PI3) kinase and protein kinase B (PKB, Akt) cascade and is fundamental to the proper regulation of β-cell survival and growth [106].

There are also reports showing that GLP-1 improves insulin-controlled glucose homeostasis in myotubes [107], [108], isolated rat adipocytes [109] and 3T3-L1 adipocytes [110]. Moreover, a study by the group of Ning [111] revealed that GLP-1 and exendin-4, can increase insulin receptor, IRS1 and GLUT4 expression levels in 3T3-L1 adipocytes. Exendin-4 is a naturally occurring analog of GLP-1 [112] that binds and activates the GLP-1 receptor with the same potency as GLP-1 [113], [114]. GLP-1 receptors are found in specific nuclei within the hypothalamus [115]. Long-term exendin 4 treatment of Zucker rats (characterized by a deficient in leptin receptors in the brain) reduces food intake and decelerates weight gain [116].

Based on the observation that GLP-1(7-36) is rapidly degraded by dipeptidyl peptidase-4, (DPP-4) yielding the inactive GLP-1(9-36) form [117], DPP-4 inhibitors have been designed to increase endogenous GLP-1(7-36) levels. One of these inhibitors is sitagliptin. DPP-4-

deficient mice are healthy, fertile, and have improved metabolic function [118]. However, as reviewed in [119], treatment of patients with sitaglipin reduced hemoglobin A1c to 0.7% and therefore there is probably no additional metabolic benefit of sitagliptin treatment compared to other hypoglycaemic agents. No definite conclusions could be drawn from published data on sitagliptin effects on measurements of beta-cell function. In addition, DPP-4 is identical to CD26, a marker for activated T-cells [120]. It has been shown that DPP-4 has several effects on immune cells, including inhibition of cytokine production, and induction of transforming growth factor-ß secretion [121]. Although short term studies in patients did not show that inhibiting DPP-4 is toxic for the immune system, more information about long term treatment is necessary especially an analysis of possible adverse effects on parameters of immune function [119].

1.4 Aims of the project

As introduced above, adipose tissue not only stores energy but also acts as an endocrine organ. It is an important hub in the regulation of blood glucose homeostasis and metabolic balance. Obesity-associated chronic inflammation and ER stress both contribute to the development of insulin resistance. While it is known that incretin hormones improve adipocyte glucose transport and lipogenesis, nothing is known about incretin hormones and the secretion of adipokines, especially under ER stress.

The aim of this project was to study if and how incretins affect the secretion of selected adipokines from differentiated adipocytes under homeostasis and ER stress.

1.5 Strategies

For the purpose described above, differentiated 3T3-L1 adipocytes were cultured in the presence of different incretin substrates (GLP-1(7-36), GLP-1(9-36), and exendin-4) or DPP-4i (Sitagliptin) under normal condition or ER stress. The accumulation of adipokines (IL-6, KC, leptin, adiponectin and TNF-α) in supernatants was assessed at different time points by multiplexing.

2 Materials and methods

2.1 Culture and differentiation of 3T3-L1 fibroblasts into adipocytes

3T3-L1 fibroblasts (purchased from ATCC, Manassas, USA) were cultured in DMEM (4.5 g/L D-glucose) medium (Invitrogen) containing 10% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were incubated at 37 °C, under 5% CO2 in a humidified atmosphere. 3T3-L1 fibroblasts were plated on gelatin-coated 24 wells plates (TPP, Switzerland) and cultured in growth medium for two to three days to reach confluence. Then differentiation medium containing 0.5 mM isomethylbutylxanthine, 1 µM dexamethasone and 1.7 µM insulin was added. Three days later, differentiation medium was replaced with growth medium, supplemented with 240 nM insulin for an additional 3 days. Cells were kept in DMEM (4.5 g/L D-glucose) containing 2% FCS for 5-7 days followed by DMEM (1.0 g/L D-glucose), 2% FCS for 5 days prior to the start of an experiment.

2.2 Induction of ER stress and incubation in the presence of substances

ER stress was induced with either 10 µg/ml tunicamycin (TU) (Applichem) or 600 nM thapsigargin (Thap) (Calbiochem). TU was dissolved at 20 mg/ml in DMSO and Thap at 5 mg/ml in Ethanol. Same amount of DMSO or Ethanol were used as the homeostasis condition. The induction of ER stress was assayed using an antibody against Grp78.

Four different substances were used in combination with ER stressors: GLP-1(7-36) amide, GLP-1(9-36) amide, Exendine-4, and DPP 4i. The final concentration of each one was 10 nM. A typical plate layout is presented in Figure 5.

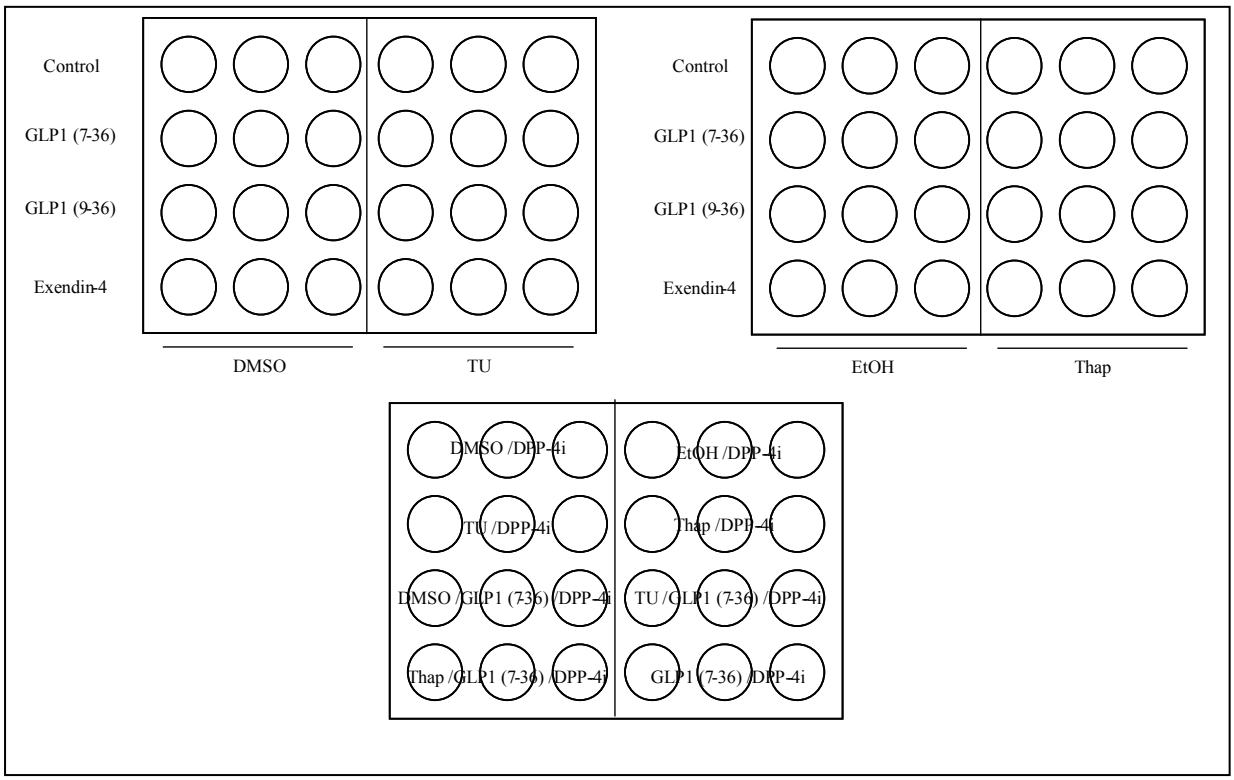

Figure 5: Outline for the incubation of 3T3-L1 adipocytes in the presence of substances.

2.3 Assessment of adipokines in supernatants

Differentiated 3T3-L1 adipocytes were incubated in medium containing the appropriate substances (as indicated in Figure 5) for 5 hours. After washing with PBS (3 times) cells were incubated in Kreb's Ringer buffer (1% BSA) with substance for 90 minutes. Supernatants were collected and marked as "c1". c1 presumably represents an early and mild condition of ER stress. Then supernatants were replaced by DMEM (1.0 g/L D-glucose), 2% FCS and respective substances for 20 hours. These supernatants were collected as "c2". Finally, after three washes in PBS the cells were incubated in Kreb's Ringer buffer containing 1% BSA and respective substances for another 90 minutes ("c3"). c2 should represent a phase with pronounced ER stress. After the collection of c3 the cells were washed in ice cold PBS three times and lysed. The induction of ER stress and the action of incretins were confirmed by Western blotting. Concentrations of adiponectin, leptin, cytokine-induced neutrophil chemoattractant (KC) and IL-6 were determined using multiplex panels from Linco Research (Labodia, Switzerland). In c3, cells already suffer from ER stress for an extended period of

time. This phase might hence be characterized by secondary effects.

2.4 Cell lysis and protein determination

After washing with ice cold PBS, cells were lysed in lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM PMSF, 0.5% Triton X-100, 10 mM NaF, 1 mM $Na_2H_2P_2O_7$, 1 mM Na_2O_4V, 3 µg/ml aprotinin, 3 µg/ml leupeptin) and incubated at 4 °C for 30 minutes with constant shaking. Lysates were transferred to Eppendorf tubes and centrifuged at 15,000 g for 10 minutes at 4 °C. Supernatants were transferred into fresh tubes and protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce).

2.5 Immunoblotting

Equal amounts of protein were loaded and separated on SDS-PAGE gels (NuPAGE, Invitrogen) and transferred onto PVDF membranes. After blocking with 2% non-fat milk (BioRad) in TBST (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 0.05 % (v/v) Tween 20) membranes were incubated with indicated primary and secondary antibodies either at room temperature for 1 hour or overnight at 4 °C. Immuno-reactive proteins were visualized by the

Lumi-Light Western Blotting Substrate (Roche) using a LAS-3000 imaging system (Fuji). Equal loading and transfer was confirmed with an antibody against actin.

2.6 Antibodies

Target	**Species**	**Dilution**	**Catalogue Number**	**Company**
Grp78	Rabbit	1:1000	sc-13968	Santa Cruz Biotechnology
Phospho-PKB (Ser473)	Rabbit	1:1000	cs 9271	Cell Signaling
PKB	Mouse	1:1000	610861	BD Bioscience
Phospho- CREB (Ser133)	Rabbit	1:1000	cs9198	Cell Signaling
Phospho-Tyrosine	Mouse	1:750	05.321	Upstate
Insulin receptor β chain (C-19)	Rabbit	1:1000	Sc-711	Santa Cruz Biotechnology

Table 2: Primary antibodies

Target	Species	Isotype	Dilution	Catalogue Number	Company
Mouse	Goat	IgG	1:3000	sc-2005	Santa Cruz Biotechnology
Rabbit	Goat	IgG	1:3000	170-6515	BioRad

Table 3: Secondary antibodies

2.7 Statistical Analysis

Data are expressed as mean ± SEM unless noted otherwise and were analyzed by unpaired two-sided Student's t-test or by Anova, where appropriate. $p < 0.05$ was considered significant.

3 Results

3.1 Introduction of ER stress and the action of incretins

Expression of the ER stress marker Grp78 was assessed by Western blotting to confirm the induction of ER stress. We also assessed expression of the insulin receptor (IRβ) and phosphorylation of PKB and CREB by Western blotting (Figure 6). Both ER stress inducers, TU (blocks glycosylation) and Thap (disrupts ER Ca^{2+} homeostasis), significantly induced ER stress. Incretins did not affect the expression of the ER stress marker Grp78. No difference was observed in expression or phosphorylation of CREB, but overall basal phosphorylation of CREB was always high. Sitagliptin reduced the expression of insulin receptor under homeostasis. We also found that tharpsigargin could enhance CREB phosphorylation significantly. PKB expression and phosphorylation were not affected by incretins or Dpp-4i.

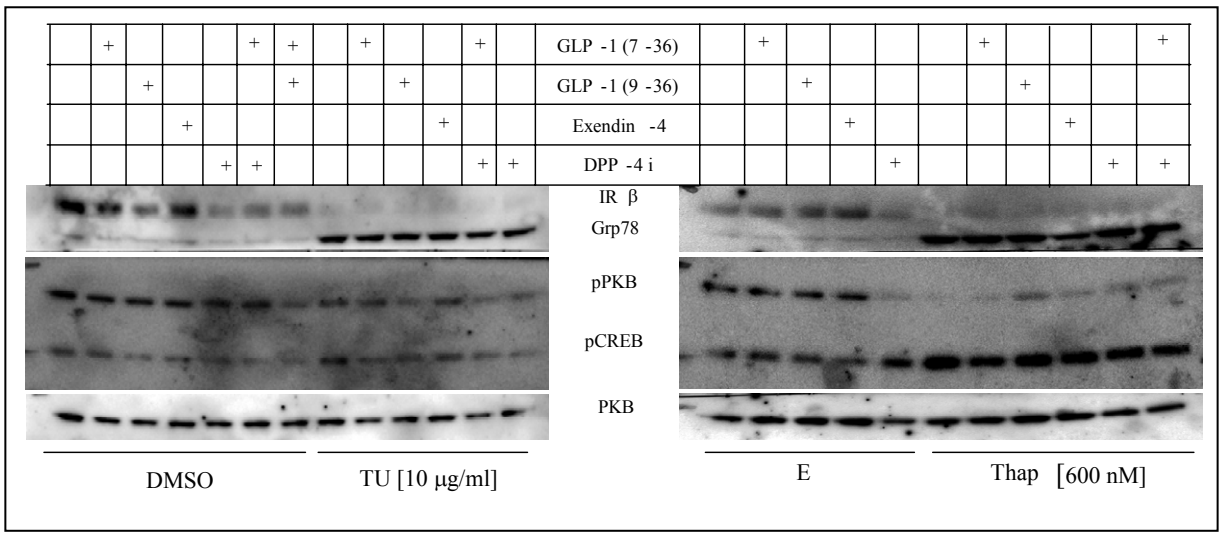

Figure 6: Induction of ER stress and incretins in 3T3-L1 adipocytes.

Differentiated 3T3-L1 adipocytes were incubated in the presence of TU or Thap as indicated, and the supernatants were collected as mentioned. After the third phase collection, cells were washed three times with PBS. Expression of Grp78/BiP, IRβ activation and expression of PKB and CREB were assessed in lysates by Western blotting.

3.2 ER stress and the secretion of adipokines

The accumulation of adiponectin, KC, IL-6, TNF-α and leptin was assessed (Figure 7). Unexpectedly, we were not able to detect TNF-α. Both tunicamycin and thapsigargin reduced adiponectin secretion significantly at c2 and c3 phases. In c1, the reduction was already visible, but not significant (Figure 7 A). Inflammatory cytokines KC (Figure 7 B) and IL-6 (Figure 7 C) were increased under ER stress. The effect of Thap was always stronger compared to TU. ER stress regulated the secretion of leptin in a time-dependent manner, increasing it at c1 and c3 phases but reducing it at c2 phase.

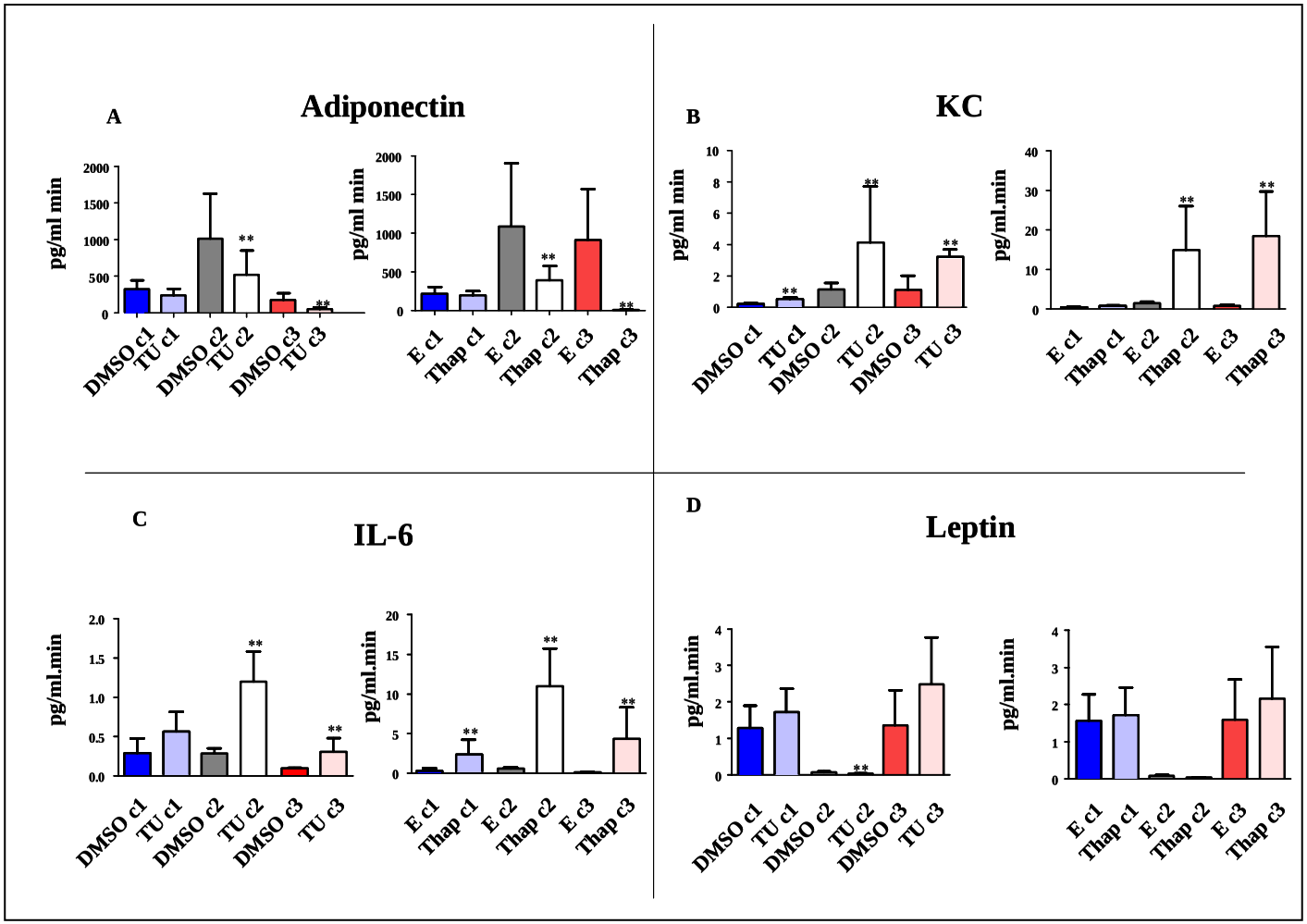

Figure 7: **ER stress changes adipokine secretion from 3T3-L1 adipocytes.**

Supernatants from c1, c2 and c3 were collected and concentrations of Adiponectin, Leptin, KC and IL-6 were determined by multiplex analysis. (A) ER stress significantly repressed Adiponectin (A) secretion but increased KC (B) and IL-6 (C) in c2 and c3 phases. ER stress reduced Leptin concentration in c2 phase (D). Data are shown in pg per min.

3.3 GLP-1(7-36)

GLP-1(7-36) induced the secretion of adiponectin up to three times under homeostasis at c2 (Figure 8 A, DMSO 199 ± 81 pg/ml vs. DMSO/GLP-1 (7-36) 628 ± 358 pg/ml (n=4, P=0.0361)). It did not change adiponectin levels under either TU- or Thap-induced ER stress (Figure 8 B, TU 562 ± 356 pg/ml (n=5) vs. TU/GLP-1(7-36) 579 ± 325 pg/ml (n=5). Figure 8 D, Thap 424 ± 197 pg/ml (n=5) vs. Thap/GLP-1(7-36) 230 ± 145 pg/ml (n=5)). At c2, GLP-1(7-36) increased KC under homeostasis (two fold) but under ER stress it had the opposite effect and attenuated the ER stress-induced elevation of KC down to control levels (Figure 9, DMSO 1239 ± 454 pg/ml vs. DMSO/GLP-1(7-36) 2497 ± 1635 pg/ml (n=5), p=0.0467. TU 4473 ± 3839 pg/ml (n=4) vs. TU/GLP-1(7-36) 1884 ± 1412 pg/ml (n=5)).

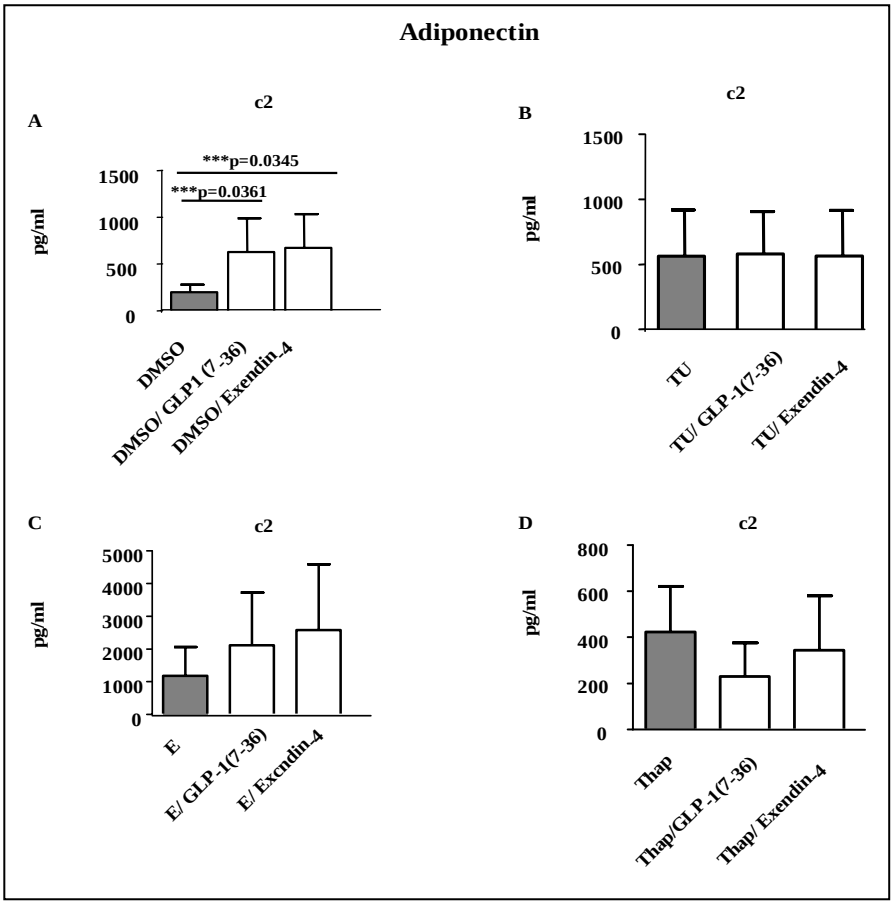

Figure 8: GLP-1(7-36) and Exendin-4 increase adiponectin secretion in c2 phase.

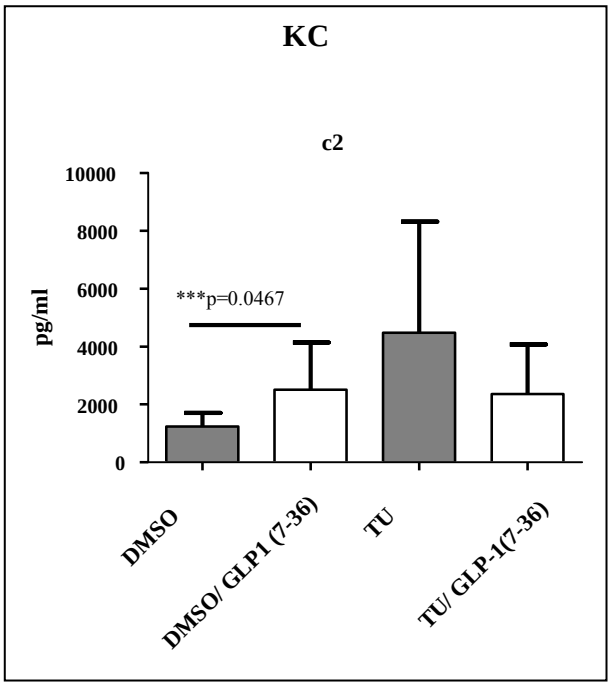

Figure 9: **The role of GLP-1 (7-36) in KC secretion of adipocytes.**

3.4 Exendin-4

Exendin-4 exerted similar effect as GLP-1(7-36) and increased adiponectin and KC secretion at c2 phase (Figure 8 and Figure 10). It increased the accumulation of adiponectin up to 3 times under homeostasis (DMSO 199 ± 81 pg/ml (n=4) vs. DMSO/Exendin-4 669 ± 364 pg/ml (n=4), p=0.0345) but had no influence under ER stress. Furthermore, Exendin-4 increased KC under homeostasis already at c1 phase (Figure 13 A, DMSO 24 ± 9 pg/ml (n=5) vs. DMSO/Exendin-4 59 ± 42 pg/ml (n=5), p=0.0143) and, as GLP-1(7-36), also at c2 phage (Figure 10 A, DMSO 1239 ± 454 pg/ml (n=5) vs. DMSO/Exendin-4 3789 ± 2706 pg/ml (n=5), p=0.0044). Exendin-4 significantly reduced KC under ER stress at c2 phase (Figure 10 B, TU 4473 ± 3839 pg/ml (n=4) vs. TU/Exendin-4 1625 ± 685 pg/ml (n=5), p=0.0094. Thap 16120 ± 11940 pg/ml (n=4) vs. Thap/Exendin-4 3019 ± 1307 pg/ml (n=4), p=0.0044).

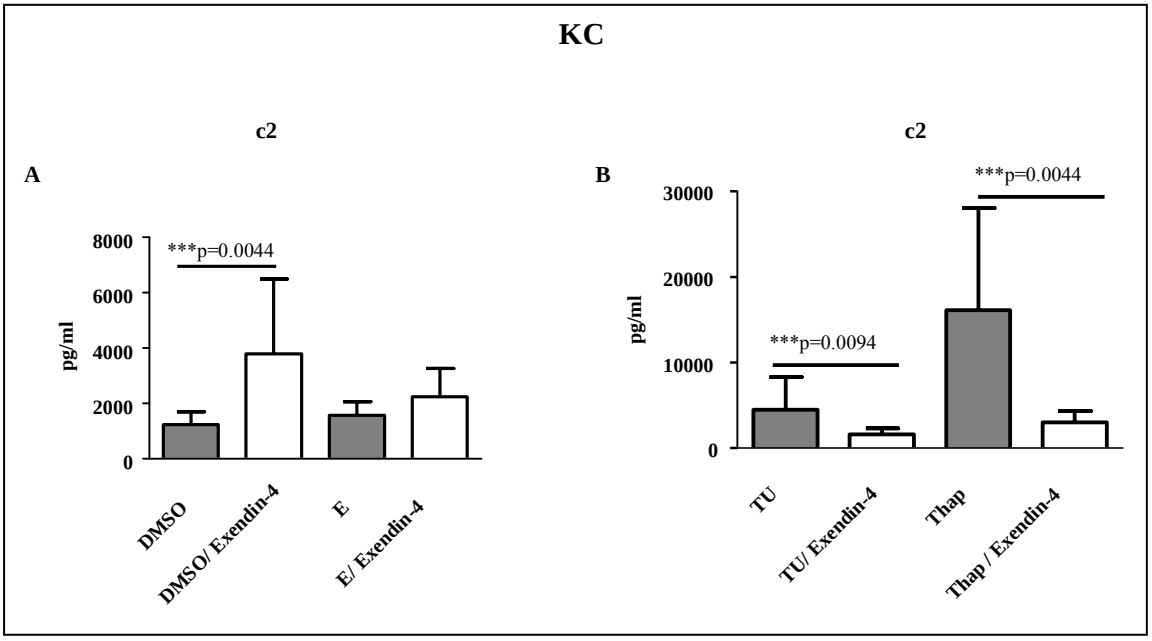

Figure 10: The role of Exendin-4 in KC secretion in c2.

3.5 GLP-1(9-36)

Under homeostasis, GLP-1(9-36) increased the accumulation of adiponectin production 2.7/2.8 times under homeostasis (DMSO/Ethanol) (Figure 11 A, DMSO 1.1 ± 0.6 µg/ml (n=5) vs. DMSO/GLP-1(9-36) 3 ± 2 µg/ml (n=4). Ethanol 1.2 ± 0.8 µg/ml (n=5) vs. Ethanol/GLP-1(9-36) 3.4 ± 2 µg/ml (n=5), p=0.0486) but had no effect on adiponectin accumulation under ER stress at c2 phase (Figure 11 B, TU 0.56 ± 0.3 µg/ml (n=5) vs. TU/GLP-1(9-36) 0.57 ± 0.33 µg/ml (n=5). Thap 0.4 ± 0.19 µg/ml (n=4) vs. Thap/GLP-1(9-36) 0.26 ± 0.16 µg/ml (n=5)). On the other hand, it greatly increased KC levels at c2 under homeostasis up to 100 times compared to DMSO control (Figure 12 A, DMSO 0.029 ± 0.01 µg/ml (n=5) vs. DMSO/GLP-1 (9-36) 2.03 ± 2. µg/ml (n=5), p<0.0001) and 5 times compared to the Ethanol control (Figure 12 C, Ethanol 0.02 ± 0.01 µg/ml (n=5) vs. Ethanol/GLP-1 (9-36) 0.1 ± 0.1 µg/ml (n=5), p=0.0041). Further more, it counteracted the accumulation of KC under ER stress at c2 (Figure 12 B, TU 0.9 ± 0.9 µg/ml (n=4) vs. TU/GLP-1(9-36) 0.41 ± 0.4 µg/ml (n=5). Figure 12 D, Thap 4.7 ± 4.6 µg/ml (n=5) vs. Thap/GLP-1(9-36) 0.056 ± 0.04 µg/ml (n=5), p<0.0001).

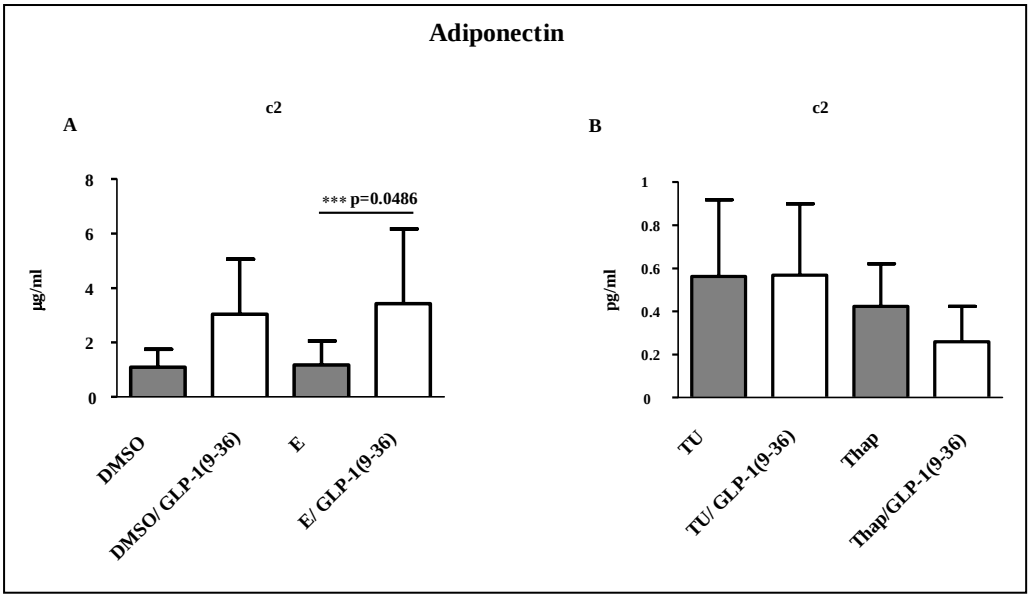

Figure 11: The role of GLP-1(9-36) in adiponectin accumulation in c2.

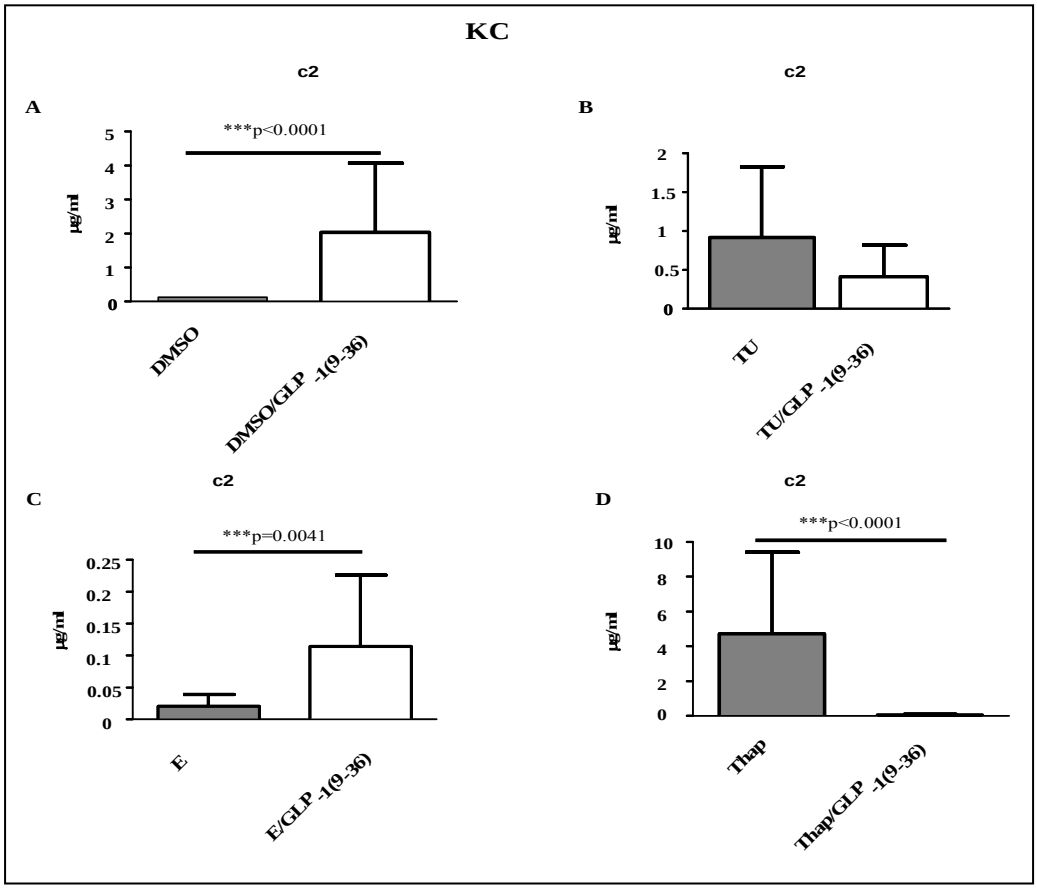

Figure 12: The role of GLP-1(9-36) in KC accumulation in c2.

3.6 DPP-4i (des-fluoro-sitagliptin)

DPP-4 inhibitors were designed to stabilise endogenous GLP-1(7-36). In our experiments, DPP-4i consistently increased pro-inflammatory factors in all three phases. Early in the c1 phase, DPP-4i increased KC under both homeostasis and ER stress (Figure 13 A, DMSO 24 ± 9 pg/ml (n=5) vs. DMSO/DPP-4i 55 ± 34 pg/ml (n=5), p=0.0293. Figure 13 B, TU 117 ± 80 pg/ml vs. TU/DPP-4i 518 ± 469 pg/ml (n=5), p=0/0048). At c2 (Figure 14) and c3 (Figure 15) phases, not only KC (Figure 14 C, Figure 15 C) but also IL-6 (Figure 14 A and Figure 15) were increased by DPP-4i under homeostasis. Under ER stress accumulation of KC in the presence of DPP4i was different in the different phases. At c2 phase, DPP-4i did not further increase KC (Figure 14 D), but at c1 and c3, KC level was significantly increased further (Figure 15 D, TU 20+-5 pg/ml (n=5) vs. TU/DPP-4i 46+-21 pg/ml (n=5), p=0.0187. Figure 15 E, Thap 386+-295 pg/ml (n=4) vs. Thap/DPP-4i 27520+-26710 pg/ml (n=5), p<0.0001).

To further analyse the effects of DPP-4i and GLP-1(7-36), we combined the two substances (Figure 16). The only changes observed were in accumulation of IL-6 and KC, relative to cells incubated in the presence of each compound alone. Synergism between the two compounds was generally not obvious. E.g.: in c2 GLP-1(7-36) alone reduced ER stress-induced accumulation of KC (Figure 9), while DPP4i even further increased it (Figure 15 D). The combination of DPP4i and GLP-1(7-36) resulted in decreased accumulation of KC in c2 (Figure 16 E), as with GLP-1(7-36) alone. The same was true with respect to IL-6 accumulation in c2 under ER stress.

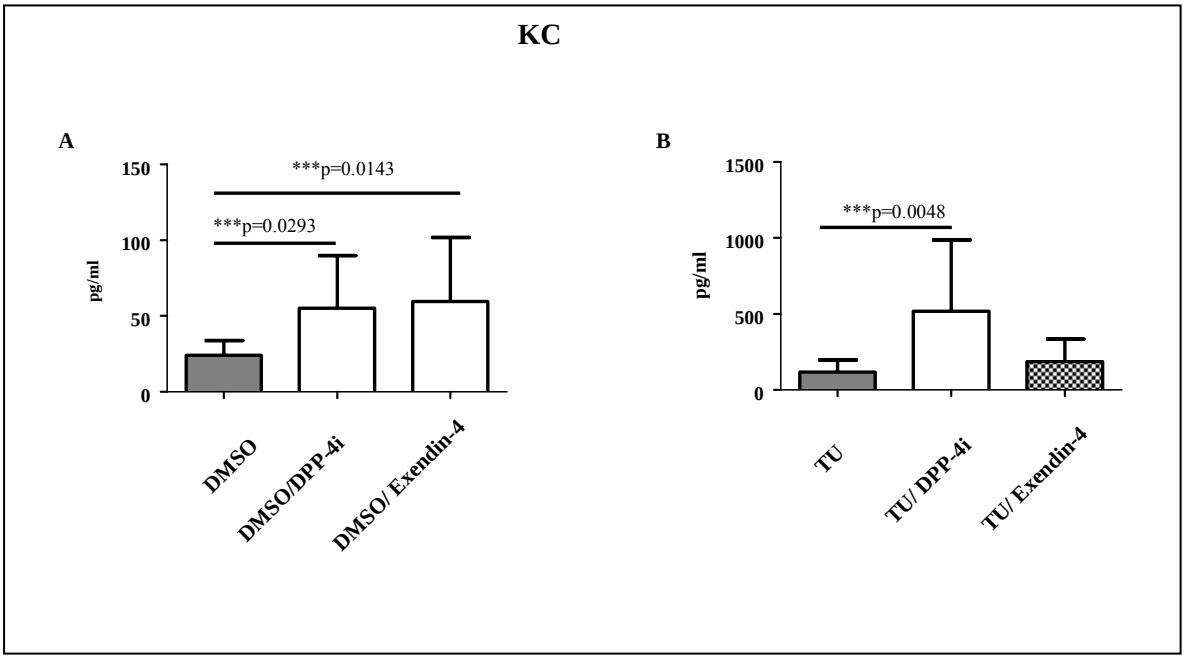

Figure 13: DPP-4i and KC accumulation in c1.

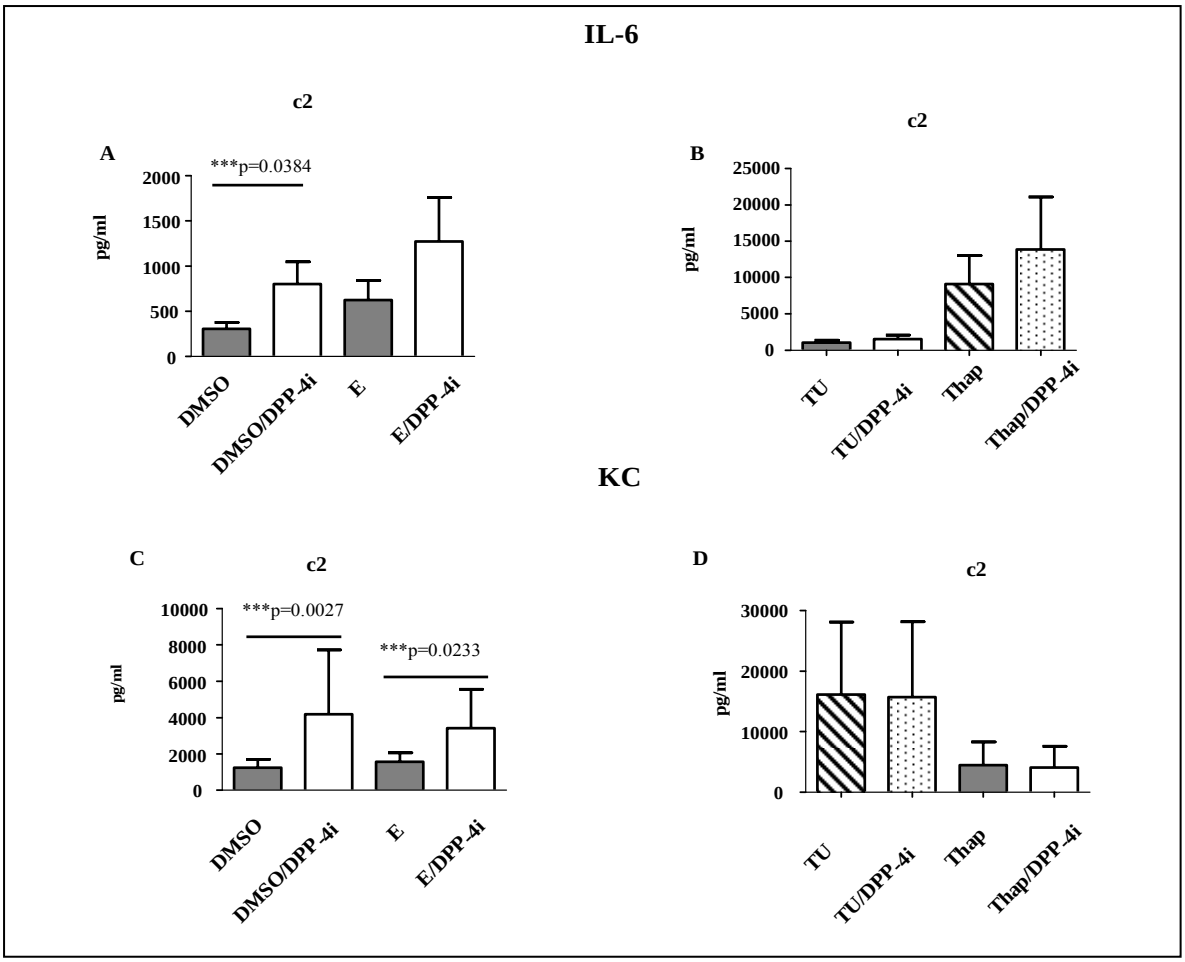

Figure 14: DPP-4i and the accumulation of IL-6 and KC in c2.

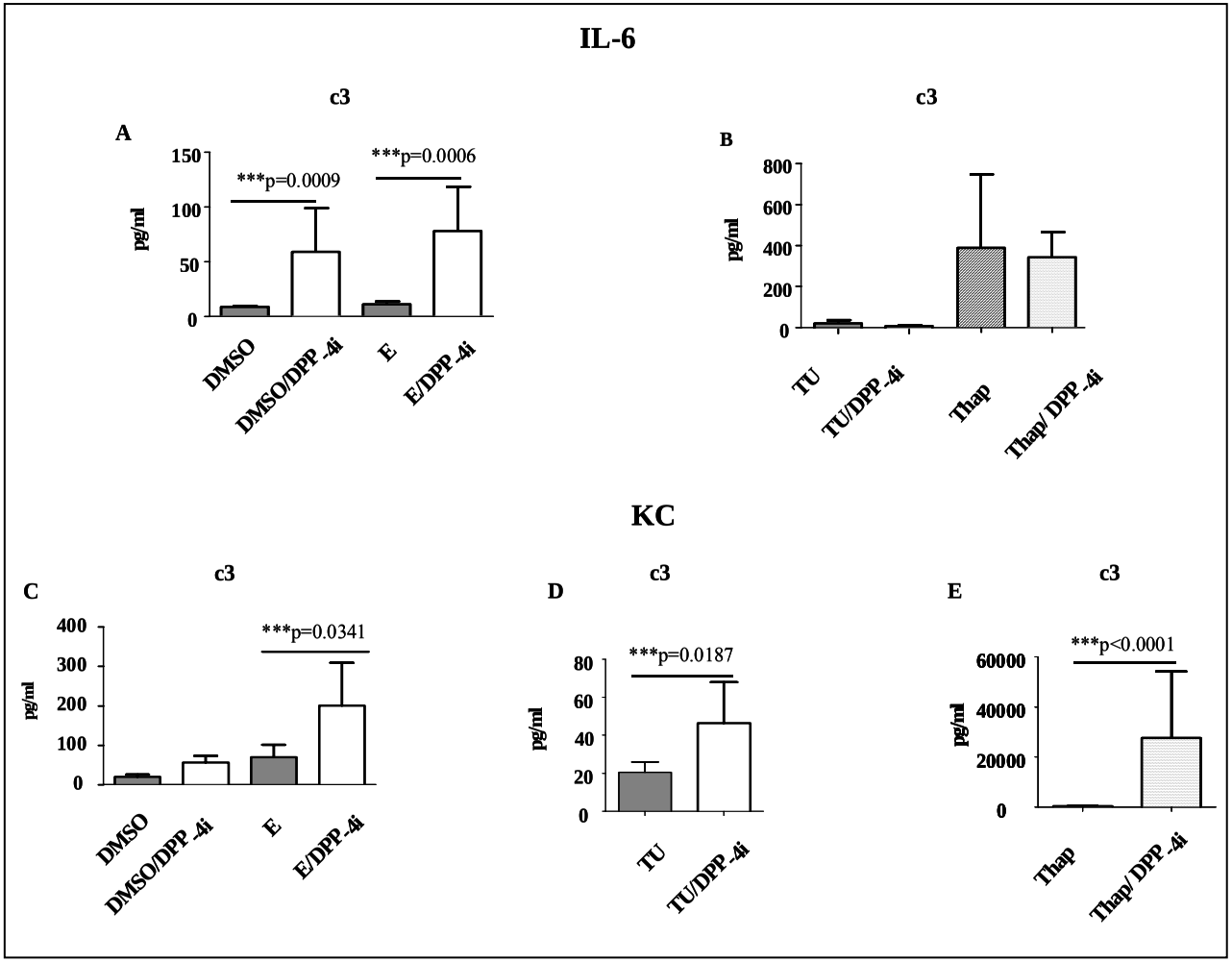

Figure 15: The role of DPP-4i in IL-6 and KC accumulation in c3.

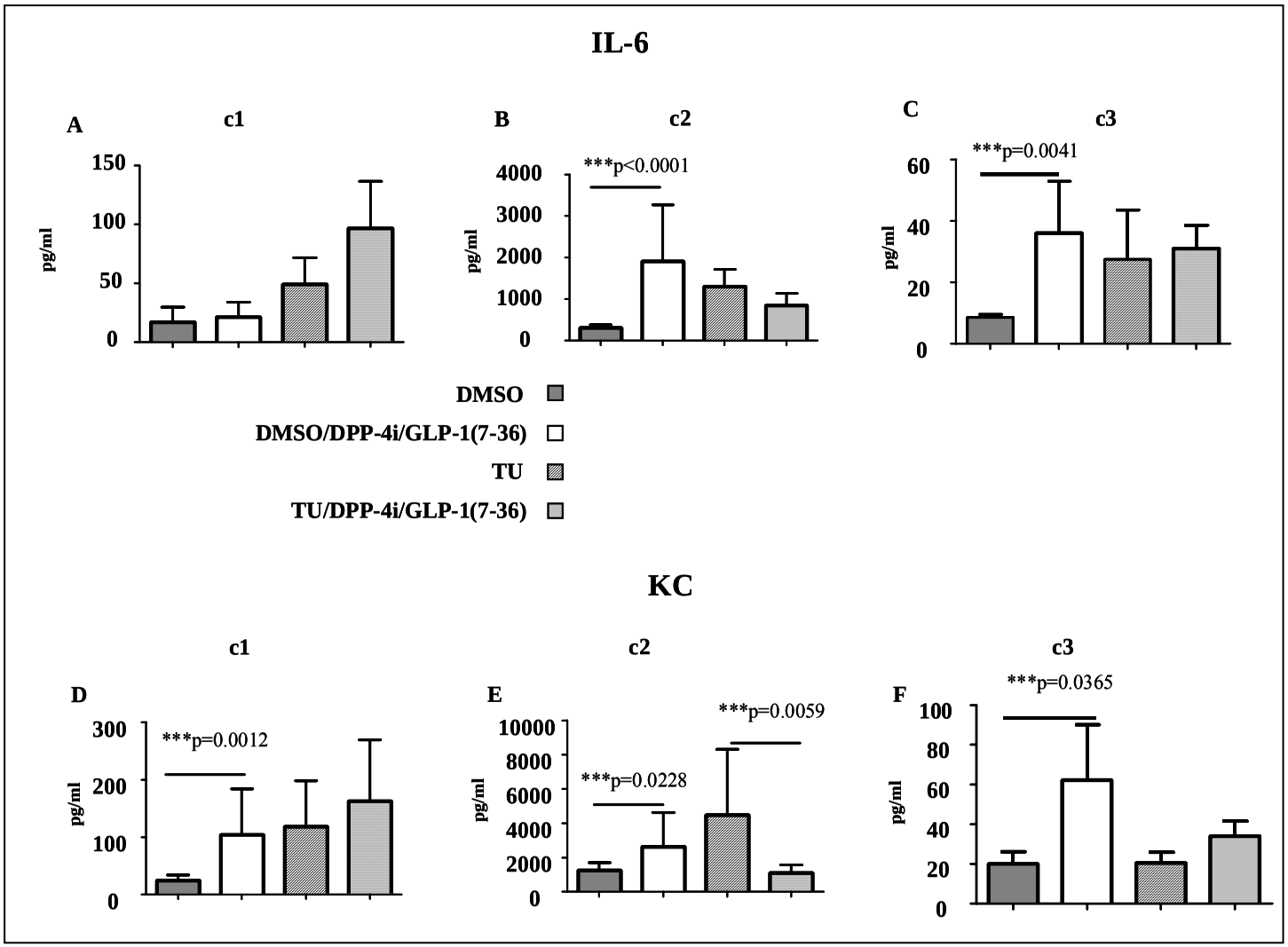

Figure 16: The role of DPP-4i/GLP-1 (7-36) in IL-6 and KC accumulation.

4 Discussion

The change in adipokine secretion during obesity plays an important role in insulin resistance and diabetes development [122, 123]. Incretins are new drug targets to improve blood glucose homeostasis in diabetes. The first incretin-based anti-diabetic treatment (exenatide; Byetta) was approved in 2005 as an adjunctive therapy in diabetic patients in whom sulfonylurea, metformin or both had failed [124]. Dipeptidyl peptidase 4 inhibitors are currently used in the clinic for the treatment of type 2 diabetes and show promising results in the improvement of glucose homeostasis. However, long-term effects have not been described yet.

In the presented thesis the role of incretins, incretin analogue and Sitagliptin in adipokine secretion was tested under homeostasis or ER stress.

4.1 ER stress increases pro-inflammatory cytokines and reduces adiponectin secretion

Previous work showed that ER stress changes adipokine secretion from adipocytes [125]. Adiponectin was strongly reduced but IL-6 significantly increased. In this study, we found that also KC production by adipocytes is elevated under ER stress. Our results fit well to the hypothesis that ER stress triggers accumulation of inflammatory factors and at the same time reduces anti-diabetic factors. Although there were already studies showing that ER stress [77] and cytokines [17] could both contribute to the development of insulin resistance in obesity, there were only few reports [126, 127] that link ER stress with cytokine production. Our study confirms and extends the notion that obesity-induced ER stress can result in adipokine secretion resembling the inflammatory situation often observed in obesity and insulin resistance.

4.2 GLP-1(7-36), Exendin-4 and GLP-1(9-36) regulate adipokine secretion in a similar manner

It is well established that GLP-1(7-36) and Exendin-4 act synergistically to improve insulin sensitivity in adipocytes [107, 111]. Our finding that GLP-1(7-36) and Exendin-4 increased adiponectin secretion under homeostasis (Figure 8, 10 and 11) supports this notion and offers an attractive mechanistic explanation. On the other hand, however, KC secretion was also elevated. Under ER stress, GLP-1(7-36) and Exendin-4 did not influence adiponectin but reduced KC levels, in line with the beneficial effects of incretins under obesity. The observation that both, GLP-1(7-36) and Exendin-4, showed similar effects indicates that GLP-1 receptor signalling might influence the secretion of adipokines.

Some studies suggested that GLP-1(9-36) may be an antagonist of GLP-1(7-36), however, other studies could not confirm this hypothesis [128]. It is still unclear whether GLP-1(9-36) is a GLP-1 receptor antagonist or even an agonist. Our finding that GLP-1(9-36) affects the secretion of adiponectin and KC in the same manner as GLP-1(7-36) (Figure 11, 13) is in support of an agonistic role of GLP-1(9-36) .

4.3 Sitagliptin induces inflammatory cytokines independent of GLP-1(7-36)

Circulating GLP-1(7-36) is rapidly degraded to GLP-1(9-36) by DPP-4. In the presence of the inhibitor of DPP-4, we expected to stabilize GLP-1(7-36). But synergism between the two compounds was not obvious. To our surprise, Sitagliptin significantly induced the accumulation of inflammatory factors but did not modulate adiponectin as found for GLP-1(7-36). It also did not change adiponectin in the presence of GLP-1(7-36) (data not shown). Changes observed in accumulation of IL-6 and KC induced by combination of Sitagliptin and GLP-1(7-39) is relative to cells incubated in the presence of each compound alone. E.g.: cells incubated simultaneously with GLP-1(7-36) and Sitagliptin (Figure 16) showed elevated inflammatory cytokines even in the c1 phase as if only using Sitagliptin. But under ER stress IL-6 was not further increased, KC was even decreased at c2 in the presence of GLP-1(7-36) or Exendin-4 alone, respectively. These results indicate that the effects of Sitagliptin on adipokine secretion are independent of blocking the degradation of GLP-1(7-36). It was known that DPP-4 not only has enzymatic function to cleave substrates such as GLP-1(7-36)

or GIP, but also acts as membrane receptor that can recruit other proteins for signal transduction into the cells [129, 130]. Effects of Sitagliptin independent of incretin action can hence not be excluded.

Previous studies have shown that DPP-4 inhibits cytokine production [121]. This finding is in line with our own observation and could explain the increased production of KC and IL-6 in the presence of Sitagliptin. Furthermore, a clinical study by Dr. Anastassios G. Pittas has shown that patients treated with Sitagliptin show an increased risk of urinary tract infection and nasopharyngitis [131]. This further supports the notion that DPP-4 inhibitors could be immuno-modulatory.

Our observation that Sitagliptin decreases the expression of insulin receptor and increases cytokine production raises the question whether Sitagliptin could induce insulin resistance. Further studies are required to confirm our observation. However, no decreased insulin sensitivity in patients treated with Sitagliptin has been reported so far.

Finally, it is hoped that this thesis has provided new useful insights into the possible importance of incretins for adipocyte biology. Although GLP-1(7-36) and Sitagliptin appear as effective agents for achieving blood glucose control, our data imply that they affect adipose tissue secretory function which might lead to yet undetected long-term systemic effects.

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EDUCATION

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LIST OF PUBLICAIONS

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3. "Differential effects of PKB/Akt isoforms on glucose homeostasis and islet mass", F. Buzzi, L. Xu, R. A. Zuellig, S. B. Boller, G. A. Spinas, D. Hynx, B. A. Hemmings, O. Tschopp and M. Niessen 2009 *"Molecular and Cellular Biology"*